PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5: A61K 37/64 — A1

(11) International Publication Number: WO 92/21373

(43) International Publication Date: 10 December 1992 (10.12.92)

(21) International Application Number: PCT/EP92/01223

(22) International Filing Date: 2 June 1992 (02.06.92)

(30) Priority data:
910100228 3 June 1991 (03.06.91) GR

(71)(72) Applicant and Inventor: LOGOTHETOU-RELLA, Helen [GR/GR]; Earos 4A, Agia Paraskevi, GR-153 42 Athens (GR).

(74) Agent: BRAUN, André; Mertengasse 5, CH-4051 Basel (CH).

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF CANPs-INHIBITORS IN PHARMACEUTICAL PREPARATIONS

(57) Abstract

The invention relates to the use of inhibitors of neutral Ca^{2+} dependent proteinases (CANPs) and their pharmaceutically acceptable addition salts or their active subunits in the field of tumour therapy, especially cancer therapy, viral diseases therapy, AIDS therapy and contraception. The inhibitors can especially be used against protease bound glycosaminoglycan-substrate dependent tumours, viral diseases and contraception by selective malignent cell killing, viruses and spermatozoa killing and inhibiting of nuclear viruscuts in a host-parasite cell system involving common protease bound substrate. The inhibitors are heatstable, tetrameric proteins at neutral pH, approximatively 240 KDa, destroyed with trypsin. The pharmaceutical compositions may be prepared in a manner known per se, with carriers or additives commonly used in the pharmaceutical industry.

USE OF CANPs-INHIBITORS IN PHARMACEUTICAL PREPARATIONS

FIELD OF THE INVENTION

The present invention relates to the pharmaceutical use of specific inhibitors of Calcium Activated Neutral Proteases (CANPs) or its active subunits. More specifically, the inhibitors or its active subunits may be especially used for the treatment of tumours, especially cancer, viral diseases, AIDS, and as anticonceptiva. The invention further relates to the manufacture of pharmaceutical preparations for the treatment of the above mentioned diseases and for the use as anticonceptiva.

BACKGROUND OF THE INVENTION

One of the most lethal properties of malignant cells is their ability to infiltrate normal tissues and to metastasize to distant areas. The normal connective tissues consist of cells embedded in an extracellular matrix containing glycoproteins, collagen, elastin and proteoglycans. There have been made suggestions that tumour associated histolytic enzymes may aid in the invasive process by removal of the matrix protein (Hart, I. et al., 1980, JNCI 64:891). Several studies have concentrated on this aspect of tumour cell biology, and increased protease production has been observed with many transformed cells (Jones P.A. and Declerk Y.A., 1980, Cancer res. 40:3222).

m- and μ-calcium-activated neutral proteases (CANPs), also known as calpain I and II, are typical intracellular cysteine proteinases of higher animals. They have been presumed to participate in various cellular functions mediated by Ca^{2+}, but their precise function are not yet clear. CANPs hydrolyse proteins of limited classes in vitro, including epidermal growth factor receptor, platelet derived growth factor receptor and protein kinase C. They

appear to be involved in regulating the turnover and degradation of muscle myofibrillar proteins and neuronal cytoskeletal elements, suggesting that CANPs are involved in essential cellular functions (Murachi T., 1983, Trends. Biochem. Sci. 8, 167-169).

In studying the role of CANPs in the above processes, several exogenous inhibitors of this enzyme have been utilized, for example leupeptin, a peptide of the structure N-acetyl-L-leucyl-L-leucyl-L-argin-al, and E64, an epoxy compound of the peptide structure L-trans-3-carboxy-oxiran-2-carbonyl-L-leucylagmatine, both specific inhibitors of thiol proteases.

Leupeptin and E64 have been proposed for use in the treatment of Duchenne Muscular Dystrophy (Hollenberg Sher J. et al., 1981, Proc. Natl. Acad. Sci. USA 78(12), 7742-7744, and Komatsu K. et al., 1986, Exper. Neurol. 91, 23-29). In addition, leupeptin and E64 have been used as pharmaceutical preparations in promoting synapse formation and innervation of muscle fibers (PCT-application WO-A-9000 401, 1990, University College, London).

In the field of cancer, only leupeptin has been used and found to inhibit the in vitro cell growth of rat brain cells only: (Nishiura I. et al., 1979, Neurol. Med. Chir. (Tokyo) 19(1), 1-8).

It is believed that the small molecules leupeptin and E64 may penetrate cell membranes and enter nerve terminals and cells and thus inhibit calcium activated proteinase (Nishiura I. et al., 1979, Neurol. Med.-Chir. 19(1), 1-8 and PCT-application WO-A-9 000 401, 1990, University College, London).

This suggested that leupeptin could be used for therapeutic purposes. However, such treatment has not been found especially successful. This is because CANPs are not

inhibited (Mehdi S. et al., 1988, Biochem. and Biophys. Res. Comm. 157(3) 1117-1123) or only partially inhibited by leupeptin (Tsuji S. and Imahori K, 1981, J. Biochem. 1990, 233-240). In addition, the substrate in malignancies upon which the inhibitors of CANPs act were unknown until now. Moreover, as will be reported later, leupeptin and E64 used in tests according to the present invention did not inhibit malignant cell growth.

It has been published that malignant cells in culture from human invasive urothelial carcinoma form tumour nodules and glycosaminoglycan membranous sacs (GSG) with membrane extensions intracellularly as well as extracellularly (Logothetou-Rella, H. et al., 1988a, Europ. Urol. 14(1), 61-64 and 65-71). The same observations were made in human trophoblast cell cultures (Logothetou-Rella, H. et al., 1989, Histol. Histopath. 4:367-374), while they were not found in human normal urothelial cells in culture (Logothetou-Rella, H. et al., 1988, Europ. Urol. 15, 259-263). The participation of GSG has also been reported in capillary formation which is enhanced in tumours in vivo (Logothetou-Rella, H. et al., 1990, Histol. Histopath. 5:55-64).

The characteristic extracellular matrix (GSG) of malignant and embryonic cells is PAS and PAS-diastase positive, identified by Papanicolaou stain by its light green colour (EA colour) and smooth, to fibrillar translucent texture. GSG in malignant cells is distributed and accumulated in intracellular and extracellular membranous sacs. The membranous GSG sacs give rise to membrane extensions which form channels through which the green GSG is passed from the inside to the outside of the cell, enhance tumour nodule formation and invade other cells in vitro.

OBJECT OF THE INVENTION

Surprisingly a new mechanism of cell to cell invasion and substrate (GSG bound CAMP) formation, common e.g. in formation of tumours, viral diseases, AIDS and fertilization were found.

Moreover it was found that administering specific inhibitors of CAMPs or an active subunit of it to provide an effective concentration of said inhibitors in human or animal body would inhibit the aforementioned processes.

DETAILED DESCRIPTION OF THE INVENTION

It was found that the intracellular and extracellular matrix (sac-GSG bound CAMPs) produced by the interaction of malignant with normal cells in vitro and in vivo can be used as substrate for the specific inhibitors of CAMPs or its active subunit.

The intracellular GSG bound CAMPs-sacs communicate with the extracellular environment with membrane extensions and form large extracellular channels (full of substrate) or diffused matrix allowing the large molecule (approximately MW 240,000) of the inhibitors of CAMPs to also enter the cells and inactivate GSG bound CAMPs. The sac-GSG bound CAMPs and extracellular matrix e.g. in tumours are produced by a new mechanism of cell to cell invasion related to that of viral cell infections and fertilization. The inhibitors of CAMPs selectively kill malignant cells only by inactivation of the intracellular and extracellular GSG bound CAMPs, a special matrix upon which viability and propagation of only malignant cells depends on.

The viral infection of cells and oocyte penetration by spermatozoa are biological phenomena which also involve cell to cell invasion, i.e. cell invasion by virus and oocyte invasion by spermatozoa, produce the same extracellular matrix (substrate) as malignant cells and require the presence of CAMPs. Based on this new mechanism

of cell to cell invasion, the inhibitors of CAMPs or its active subunits also exhibit antiviral and contraceptive action.

A possible mechanism of the action of an inventive inhibitor on malignant cells might be e.g. the dissociation of the inhibitor tetramer, upon contact with extracellular GSG (substrate) bound CAMPs, into subunits (e.g. monomers, the MW of which vary considerably and depend on the substrate used) and formation of inactive inhibitor-proteinase complex (blue hematoxylinophic granules).

Then the following events may be taking place. The inhibitor subunit produced extracellularly may diffuse through malignant cell membrane and inactivate the endogenous CAMPs or the intracellular activated CAMPs diffuse extracellularly towards a lower concentration gradient (after the inactivation of the extracellular activated CAMPs) and get inactivated by the extracellular inhibitor monomer. Also both events might be taking place. Empty cytoplasmic vacuoles observed in inhibitor treated malignant cells support the diffusion of activated CAMPs and its inactivation extracellularly.

Moreover the extracellular GSG-CAMPs channels are large enough for passage of the inhibitor all the way into the GSG-CAMPs sacs.

The effect of the inhibitors of CAMPs on spermatozoa suggests that spermatozoa are associated with the CAMPs enzyme, upon which their motility, viability and penetration ability depends. The increased sperm motility observed with high Ca^{2+} concentration (Fakih et al., 1986, Fertil. Steril. 46(s), 938-944) could be achieved via the activation of sperm endogenous CAMPs. The high Ca^{2+} release by the egg cortex upon polyspermy prevention (Steinhardt et

al., 1977, Develop. Biol. 58, 185-196) might involve the participation of the oocyte's possible inhibitors of CAMPs.

The cytologic effect of the inhibitors of CAMPs on semen mucin strongly documents that the active enzyme is bound to glycosaminoglycans, an apparently common substrate for both the protease and inhibitor action. Again the mechanism of action of the inhibitors on spermatozoa might be the dissociation of a certain inhibitor upon contact with mucin (substrate) bound CAMPs and formation of inactive inhibitor -proteinase complex (blue hematoxylimorphic granules). The inhibitor subunit produced extracellularly after dissociation, is probably diffused through spermatozoa membrane and inactivates the endogenous CAMPs or CAMPs moves extracellullarly towards a lower concentration gradient after inactivation of extracellular CAMPs. The inhibitors of CAMPs being non-toxic to normal cells and toxic to spermatozoa seems a promising male contraceptive agent.

This observation enables a new approach to many pharmaceutical problem, but especially enables the manufacture of new pharmaceutical preparations containing suitable inhibitors of CAMPs for the treatment of tumour, especially cancer, all kind of viral deseases, AIDS and fertilization and methods for the treatment of the aforementioned pharmaceutical diseases and needs.

The inhibitor of CAMPs used in the invention is preferably a tetrameric protein of MW of approximatively 240,000, based on its elution from Sephadex G-200, heatstable at neutral pH, destroyed on digestion with trypsin, and dissociated into its subunits of a MW of approximatively 60,000 by 0.1-1 mM Ca^{2+}, based on SDS-polyacrylamid gel electrophoresis (as described by Melloni et al. in Arch. of Biochem. and Biophys. Vol. 232, No. 2, 513-19, 1984). All pharmaceutical acceptable salts, derivatives, analogues or active subunits of different MWs (which depend on the

substrates used) thereof can also be used as specific inhibitors of CAMPs.

It is believed that the MWs of the active subunits of the specific inhibitor depend on the substrates (e.g. Casein, denatured globin etc.) used. Therefore, the MW of the active part of the inhibitor may be higher, e.g. approximatively 150,000, or lower, e.g. approximatively 15,000, as indicated above.

The inhibitors may be endogenous native inhibitors isolated from a biological source, like erythrocytes, brain, cardia muscle, lung, spleen, liver, skeletal muscle, kidney, testis or the like, and optionally purified, but especially from rabbit skeletal or liver.

A preferred inhibitor isolated from rabbit skeletal is manufactured and sold by Sigma Chemical Company, St. Louis, USA, under the product number P-0787.

The inhibitors or active fragments thereof, like subunits of MG 60,000, may also be produced synthetically, especially by bio- or gentechnological methods, e.g. by expression in Escherichia coli.

In the present invention the pharmaceutical preparation may be in the form of a solution, powder, injection, tablet, capsule, pellets, in a fast or sustained release form, each containing a suitable amount of a specific active or synthetic and eventually purified inhibitor or its pharmaceutically acceptable addition salts, active subunits, fragments, derivatives, or related compounds together with well-known suitable excipients.

The inhibitors may preferably be administered to humans and warmblooded animals intramuscularly, subcutaneously, intraperitoneally or intravenously in an amount which

depends on the kind and severity of the disease, the inhibitory effect of the inhibitor, the route of administration, the species to be treated, the weight and the general condition of the patient, and has in most cases finally to be decided by the responsible physician. In general the dose is between about 1 mg/kg per day and 25 mg/kg per day. However, if need be also higher doses, e.g. up to 100 mg/kg per day, may be administered.

The surprising effect of the inhibitors of CAMPs have been confirmed and verified by the following tests, whereby all tests have been fulfilled also with Aprotinin (Sigma A-4 529), Trypsin-Chymotrypsin inhibitor (Sigma T-9777), Leupeptin (Sigma L-2884) and E64 (Sigma E-3132) dissolved in RPMI-1 640 with 25 mM hepes as control protease inhibitors. All inhibitor solutions were filtered through 0.22 µ Sartorius filters, dispensed in aliquots and frozen at -20° C. Fresh or thawed inhibitor solutions were used.

However, the surprising effect was only performed by the inhibitors of CAMPs according to the invention. In the following tests the brownish tan powder of the inhibitor of CAMPs (Sigma Chemical Company, St. Louis, USA, product number P-0787), 50 U/645 mg solid from rabbit skeletal muscle, was dissolved in 5 ml plain RPMI-1640 with 25 mM hepes (Seromed), resulting to a clear tan solution (10 U/ ml), whereby one unit (U) is that quantity of inhibitor which will reduce the activity of 1 unit of CAMPs (Sigma Chemical Company, product number P 4533) by 50 % at pH 7.5 at 30°C (reaction volume = 1.8 ml, 1 cm light path). Of course, the scope of the invention shall not be reduced to the use of the concrete inhibitor used in the following examples.

EXAMPLE 1

The use of the native Inhibitor of CAMP for inhibiting growth and viability of malignant cells in Vitro

Cell culture establishment

Stationary cell cultures were established from human solid tumour tissue specimens by enzymatic digestion. Malignant lung cell lines from metastatic lung carcinoma, M-cells, P-cells and P-cells have recently been characterized by the applicant. Malignant urothelial cell cultures were established from tissue specimen from patients with invasive transitional cell carcinoma. The five established urothelial malignant cell lines were designated as Pa-cells, R-cells, S-cells, Br-cells and IG-cells. Only the patient where Pa-cells derived, had received bladder intravesical infusions of anticancer drugs. Melanoma cell culture (Ha-cells) was originated from a male patient who suffered from primary rectal melanoma, metastasized at the lymph nodes of the right arm where tissue specimen was obtained.

Malignant bone marrow cells were originated from bone marrow aspirates from five (5) patients with chronic myeloid leukemia. Walker tumour rat cells were isolated from transplanted tumour tissue into Wistar rats. Normal human liver cells (L-cells) were isolated from liver tissue specimen from a male patient who underwent surgery for the removal of his gall bladder.

Normal fallopian tube cells (F-cells) were isolated from tissue specimen from a female patient who underwent total hysterectomy. Normal bladder cells (N-cells) have been characterized previously (Logothetou-Rella, H. et al., 1988, Europ. Urol. 15, 259-263). White blood cells from five healthy persons were also used as control cells.

Mice embryos harvested at the 2-cell stage were cultivated in complete Earle's balanced salt solution (EBSS

supplemented with 10% foetal bovine serum and antibiotics) to the stage of hatched blastocysts. When the embryonic cells were all out, the culture was used for cytology. Amniotic ebryonic cells from five (5) pregnant women cultivated for prenatal diagnosis were also used in this study. All cell cultures were grown in complete medium, RPMI-1640 (Seromed) supplemented with 10% foetal bovine serum (Seromed), glutamine and antibiotics (Seromed), incubated at 37 °C in a CO_2-humidified incubator. Stock cells are stored frozen in liquid nitrogen.

Cytogenetic analysis

Chromosomal analysis of M-cells, P-cells and B-cells have recently been reported (Logothetou-Rella, H. et al.,1991, J.Exper. Clin. Cancer Res., submitted for publication). Urothelial malignant, Pa-cells consisted of malignant cell clones only, with polyploidies up to 147 chromosomes and complex structural abnormalities. S-cells consisted of malignant cell clone with regular tetraploidies, up to 20% of the cell population and 80% normal cell clone. Br-cells consisted of normal and malignant cell clones but detailed chromosomal analysis was unsuccessful. Melanoma Ma-cells revealed only double minutes. Liver L-cells, fallopian tube F-cells, and amnictic embryonic cells were cytogenetically normal.

Two techniques were used to determine the inhibitor's cytotoxicity on tumor and normal cells.

a) Cytologic changes of cell culturer in continuous presence of the inhibitors

Seven kinds of complete medium RPMI-1640 were prepared. One was supplemented with 1 U/ml of the inhibitor of CANPs; the second with 2 mg/ml trypsin-chymotrypsin inhibitor; the

third with 1 mg/ml aprotinin; the fourth with 1mg/ml leupeptin; the fifth with 1mg/ml E64; the sixth with all five inhibitors at the same concentration and the seventh complete RPMI-1640 as a control medium.

Ten glass petri-dishes (5 cm diameter) were seeded each with $1x10^6$ M-cells and another ten dishes, each with $1x10^6$ P-cells. Duplicate cell cultures received each kind of complete medium containing the inhibitors and control cultures containing only complete medium. The cell cultures were incubated at 37°C in a humidified CO_2-inhibitor for 120 hours. The culture medium was changed with a fresh one of the same kind in each case, 24 and 72 hours after culture initiation. Half of the cell cultures were fixed in 50% ethanol 72 hours and the other half 120 hours after culture initiation. All cell cultures were stained with the Papanicolaou method.

Post confluent stationary cell cultures of malignant M-cells, P-cells and normal L-cells (20 days of continuous cultivation) that had produced abundant extracellular matrix, received fresh complete medium RPMI-1640 supplemented with 1 U/ml of the inhibitor of CAMPs and incubated at 37°C for 3 days, then fixed in 50% ethanol and stained with the Papanicolaou method.

Trypsin-chymotrypsin inhibitor, aprotinin, leupeptin and E64, did not affect the growth and cytology of M- and P-cells as compared to control cell cultures.

The inhibitor of CAMPs caused great exfoliation of cells and extracellular matrix (ECM) in the culture medium, after 72 hours of continuous presence in cultures. All exfoliated cells were dead (according to trypan blue stain) consisting of hyperchromatic, pyknotic nuclei, little cytoplasm and nuclei with tails. On the culture dish surface, a few, countable per field, attached fibroblast-like cells

remained alive, cytologically normal. All other cell culture dishes (except cases No. 1 an No. 6 which contained the inhibitor) and the control ones were full of cells and nuclear vlimma (="NV", "vlimma" = bullet; state of a parasitic cell after numerous dividing operations using a host cell, where it reaches the size of a nucleolus with a nuclear head and an attached tail resembling a small immotile spermatozoo. The production of nuclear heads, the shooting and implantation in other cells occurs mainly in culture areas, where extracellular glycosaminoglycans bound CANP and cell membranes are located. In normal cell cultures NV are not found, whereas it was observed e.g. in human solid and hematologic tumours free or during production still attached to the mother cell. NVs are end cell products of incomplete, unequal, asymmetrical division of malignant cells. Upon their production they eventually detach from their mother cell and seek a host cell at random. When NVs are implanted and incorporated in the nucleus of a normal host cell, it can be considered as a process similar to fertilization or viral infection. As a result, the host cell's genotype and phenotype is altered and behaves like a transformed cell. After many divisions, the host cell looses its cytoplasm and cannot divide itself anymore; it needs support by another host cell or extracellular matrix, thus forced to become a parasite and produce NVs.) uncountable per field, without cell exfoliation, with macroscopically apparent green, fibrillar, translucent ECM and GSG sacs. The observations were persistent after 120 hours of continuous presence of the inhibitor of CANPs in cell cultures, except that the survived fibroblast-like cells had grown up in the presence of the inhibitor of CANPs.

Post-confluent M- and P-cell cultures exhibited cells with vacuolated cytoplasm as a strain, and degenerated nuclei of different sizes with and without tails. The rounded up, detached, dead cells were holding to each other on the

culture dish surface by a network of hematoxylinophilic (blue) membranes visible microscopically. The DCM and GSG sacs had disappeared. Instead large masses of hematoxylinophilic granules were present visible microscopically.

b) Liquid medium short-term culture method (Chang S.Y. et al, 1989, Eur. Urol. 16, 51-56).

The cells were detached with trypsin-EDTA (Seromed) resuspended in complete RPMI-1640 and cell counts were made using a hemocytometer. Viable counts were assessed using the 0.4% trypan blue exclusion method. The cells were then washed once with complete RPMI-1640, centrifuged at 200 g for 8 min, resuspended in complete RPMI-1640 at 30,000 -200,000 cells per 0.5 ml medium and inoculated in polypropylene tubes as shown as follows:

Test tube case No.	nCAMP inhibitor (U/ml)	Volume (ml)			
		nCAMP inhibitor	Cell suspension	F.B.S.*	Complete RPMI-1640
1	1	0.1	0.5	-	0.4
2	2	0.2	0.5	-	0.3
3	3	0.3	0.5	-	0.2
4	4	0.4	0.5	0.05	0.05
5	5	0.5	0.45	0.05	-
6	6	0.6	0.35	0.05	-
7	0	0.0	0.5	-	0.5

*F.B.S. = Foetal bovine serum

Duplicate samples of cells were tested for each concentration of the inhibitor. All samples were incubated and shaken in a water bath at 37°C for one hour. Then the cells were washed twice with complete RPMI-1640 by centrifugation at 200 g for 8 min. Each rinsed cell pellet was resuspended in 1 ml complete RPMI-1640, the cells were then rendered single by gentle pipetting and were then seeded in 24-well microplates (Costar Cambridge Mass.) for a 4-day period of short term culture at 37°C under a humidified atmosphere of 5% CO_2. The cytotoxicity assessment was done using the dye exclusion method of 0.4% trypan blue. The degree of cytotoxicity was measured according to the following formula:

$$\text{Cytotoxicity (\%)} = 1 - \frac{\text{Number of viable cells in the experimental group}}{\text{Number of viable cells in the control group}} \times 100$$

The inhibitor of CANPs selectively killed all kinds of malignant cells tested (Table 1) while allowing normal cells within the same or separate culture to grow and propagate (Table 2). The optimum concentration of 4-5 U/ml inhibitor killed all malignant clones, while lower concentration killed lower percentage of malignant cells. Higher concentration did not alter the results. The inhibitor was not cytotoxic to normal cells including liver cells, fallopian cells and WBCs. Cytogenetic analysis of the survived cells (in mixed cell lines) after the inhibitor CANPs treatment showed normal karyotype. The inhibitor of CANPs was also cytotoxic to embryonic cells.

Table 1. Categories of cases with various cells tested.

Tissue origin	Designation	Malignant and normal clone	Malignant clone	Normal clone
Bladder transitional cell carcinoma	Pa-cells		+	
	Br-cells	+		
	S-cells	+		
	IG-cells		+	
	R-cells	+		
Lung carcinoma	M-cells	+		
	P-cells	+		
	B-cells	+		
Melanoma	Ma-cells		+	
Chronic myeloid leukemia	BM-cells		+	
Walker rat tumor	W-cells		+	
Normal liver	L-cells			+
Normal urothelium	N-cells			+
White blood cells (5 specimens)	WBC			+
Human amniotic embryonic cells (5 specimens)				+
Human fallopian cells (F-cells)				+

+: Indicates the cytogenetic state of each cell type.

Table 2. Sensitivity of cells to different concentrations of the inhibitor of CAMPs.

Tested cells	Inhibitor-nCAMP (U/ml)		
	1	4	5
M-cells	24	65	65
P-cells	45	82	
B-cells	86	87	
Pa-cells	24	99	100
S-cells		34	
Br-cells		55	
R-cells		83	
IC-cells		100	
Ha-cells	21		100
Walker tumor cells		100	
Malignant bone marrow cells		88-100	
N-cells	0	0	0
L-cells	0	0	0
WBCs	0	0	0
Embryonic cells	45	95	100
F-cells	0	0	0

Inhibitor cytotoxicity (%)

The cytotoxicity of the inhibitor in each specimen was obtained from mean of duplicate samples.

EXAMPLE 2

Use of the inhibitor of CAMPs on the viability of normal and malignant urothelial tissues

Tumour (from 5 patients) and normal (from 5 persons) tissue pieces of human urothelium of 2 mm x 2 mm x 2mm size were rinsed in complete RPMI-1640, handled gently with fine forceps, immersed one piece (of each type of tissue) in complete RPMI-1640 (control) and one piece in the inhibitor solution (10 U/ml) in polypropylene tubes and incubated at 37°C for one hour in the humidified, 5% CO_2 incubator. All tissue pieces were then rinsed carefully in complete medium and were immersed in polypropylene tubes (1 piece/tube) containing 2 ml complete RPMI-1640, and then incubated for 4 days at 37°C. The tissue pieces were fixed in formaldehyde, embedded in paraffin and tissue sections were stained with eosin-hematoxylin. The exfoliated cells in the tubes with the malignant tissue pieces were allowed to settle in a conical polypropylene tube for 10 min, then smeared on glass slides fixed with cytospray and stained with Papanicolaou. The inhibitor of CANPs caused massive cell exfoliation of the malignant tissues. Histologic examination of the inhibitor treated malignant tissues exhibited bionecrotic to necrotic areas and large tissue areas consisting of eosinophilic extracellular matrix denuded of cells. The exfoliated cells were dead, with degenerated nuclei, and spermatozoa-like morphology, separated from each other and lacking the green ECM. The very few malignant tissue exfoliated cells, in the absence of the inhibitor of CANPs, showed compact cell masses in green ECM with indiscrete cell boundaries.

Normal urothelial tissues were kept intact after treatment with the inhibitor.

EXAMPLE 3

The use of the inhibitor of CANPs against human tumour nodule in vivo

A female patient with breast metastatic carcinoma, with extensive hepatic bone and subcutaneous metastasis, after repeated chemotherapy and radiation treatment without success, and also in a general bad health condition, approved the trial of the drug in her subcutaneous nodules. Nodules were located all over the chest and some in the abdomen. One hard nodule the size of a pea was injected in its center with 0.1 ml (1 U/0.1 ml) inhibitor of CAMPs dissolved in RPMI-1640 with 25 mM hepes. Twenty four hours later the treated nodule and a nearby untreated control nodule were removed, fixed in formalin and embedded in paraffin for microscopical examination.

The patient did not show any allergic reaction 1, 4 and 24 hours after the injection. The nodule feeling was softer and slightly smaller 4 hours after the injection. Twenty four hours later the nodule was soft and reduced to half its originial size. The treated neoplasm was histologically characterized by degenerated small cellular aggregation and many degenerated single cells. Most of the cells had irregular pyknotic, karyolytic or degenerated vacuolated nuclei. Some cells showed vacuolated cytoplasm. In the outer peripheral area of the tumour there remained a few neoplastic cells with regular nuclei, fine chromatin and slender single nucleoli. The main area of tumour cell degeneration, caused by the inhibitor of CAMPs, was measured approximately to a total of 3.4 mm x 2.5 mm out of the 5.2 mm x 3.5 mm main tumour section area. Neighboring sweat glands and the overlying epidermis were kept intact. There was no inflammatory reaction not even around the fissural hemorrhagic area caused by the injection. Histologic examination of the tumour nodule without treatment showed that the neoplasm was characterized by viable large columns or single strands of neoplastic cells with relatively uniform ovoid or roundish nuclei with finely stippled chromatin and slender nucleoli. Histologic picture was compatible with metastatic breast carcinoma.

EXAMPLE 4

The use of the inhibitor of CAMPs against rat tumours in vivo

Two Walker tumours were excised 2 weeks following the subcutaneous implantation of tumour tissue in Wistar male rats. Tumour cell suspension for injection was prepared as described previously (Fisher E.R. and Fisher B., 1959, 12, 926-928). A group of Wistar male rats, weighing 100 g each, were injected with $10x10^6$ Walker tumour cells subcutaneously in the left foot pad. The rats were then divided into four groups, two control and two treated. Treatment was initiated when tumours had reached a measurable size of 50-100 cu.mm. The first group of rats was injected intraperitoneally, each rat with 50 U/2.5 ml (645 mg/2.5 ml) inhibitor of CAMPs, once a day, for a period of 2 days (0.5 U/kg or 6.45 mg/kg rat body weight).

The second group of rats was treated intraperitoneally, twice daily for 5 days with the dose of 0.25 U/kg (3.23 mg/kg) rat body weight. Control rats were injected each with 2.5 ml Medium RPMI-1640 with 25 mM hepes. All rats were sacrificed 4 days after the last treatment for the injected legs, of the control groups, were all covered with tumours including up to the shoulder blade and accurate control tumour measurements were impossible. The tumour-legs, lymph nodes and liver from all rats were excised, fixed in formalin and embedded in paraffin for histologic studies. Tumour volumes were measured every day after the first dose, with calipers. The inhibitor of CAMPs caused 50% tumour regression in the first group of treated rats and 90% in the second group. All groups (treated and control) started at time 0 without any significant difference in tumour volume.

The rats under treatment were healthy and did not show any allergic reaction or side effects to the high dose of the inhibitor originating from rabbit skeletal muscle. Histologic examination of livers of the treated rats did not show any cytotoxic effects caused by the inhibitor, as central venules were observed without necrosis or cellular damage.

Among the first treated group one rat developed metastatic abdominal focus and another one metastatic hepatic focus. The feeling of the abdominal focus disappeared 24 hours after the first dose. Histological examination showed necrosis of large carcinomatous nodule with formation of abscess, necrosis of the overlying epidermis and ulceration. The liver metastatic focus was necrotized, exhibiting necrotic material with nuclear debris in the center and remnants of carcinomatous tissue with mitoses in its periphery. The foodpad tumours of treated rats showed necrotic areas of variable size with formation of microabscesses. These results become more important if taken into account the aggressiveness of Walker tumor cells, (rats usually die 20 days after tranplantation).

CONCLUSION DEDUCED FROM EXAMPLE 1-4

The pharmaceutical composition, of the present invention killed all malignant cells of different chromosomal abnormalities, tissue and species origin without effecting normal cells' genotype.

The best in vitro dosage being the one containing 4-5 units of the inhibitor of CAMPs per milliliter of solution.

This pharmaceutical composition was not cytotoxic to normal cells including liver and WBCs. It exhibited a broad spectrum of action on different types of human tumours. It

was cytotoxic to human solid and hematologic tumour cells and even more to chemoresistant tumour cells (lung P-cells and bladder Pa-cells) of different tumour origin (embryonic cells were very sensitive to the inhibitor because of their resemblance to malignant cells).

The pharmaceutical composition of the present invention tested on rat tumours caused 50-90% regression of the main tumours, inhibited metastasis and caused necrosis of metastatic foci. It was non-immunogenic, non-toxic suitably used in a daily dose of 0.5 U/Kg, (6.5 mg/Kg) of body weight in single or divided doses. The administration is suitably continued until complete tumour regression.

EXAMPLE 5

Contraceptive action of the inhibitor of CAMPs

Fertile motile sperm (after swim up test) from 10 donors, was dispensed in 4 plastic (5 ml, Falcon) test tubes each. Two test tubes received 0.4 ml sperm suspension (600,000 spermatozoa) and 0.6 ml complete EBSS. The other two test tubes each received 0.4 ml sperm suspension, 0.4 ml (10 U/ml) inhibitor of CAMPs and 0.1 ml foetal bovine serum. All tubes were incubated at 37°C for 1 hour. The spermatozoa were then washed twice with complete EBSS and centrifuged. The sperm pellets were resuspended each in 2 ml complete EBSS and incubated at 37°C for 18 hours, at which time spermatozoa were counted by the Eosin Y viability exclusion stain, smeared on glass slides, fixed in 50% ethanol and stained with Papanicolaou.

The degree of cytotoxicity caused by the inhibitor of CAMPs was measured according to the following formula:

$$\text{Cytotoxicity (\%)} = 1 - \frac{\text{Number of viable inhibitor treated spermatozoa}}{\text{Number of viable spermatozoa of control samples}} \times 100$$

Inhibitor treated and non treated sperm was inoculated in postconfluent granulosa cell cultures, incubated at 37° C for 18 hours, fixed in 50% ethanol and stained with Papanicolaou. Motility counts of the inhibitor treated motile sperm showed only 3% motile sperm, 2 hours post treatment and no motile sperm 18 hours post treatment. Eighty percent of the immotile sperm were dead (stained with eosin Y). Inhibitor treated sperm failed to penetrate granulosa cells during co-cultivation.

Cytologic examination revealed that 80% of the inhibitor treated spermatozoa, 18 hours post treatment, had coiled tail ends and clear acrosome caps. Moreover, the green fibrillar mucin present in the control samples was changed to dispersed loose masses of large blue granules in the inhibitor treated spermatozoa.

EXAMPLE 6

Antiviral action of the inhibitor of CAMPs in vitro

1) On Epstein-Barr Virus (EBV)

Viral infection of cells which involves host-parasite interaction. Viruses are also vehicles of biological active DNA or RNA into host cells in order to survive and propagate, causing viral diseases to animals and humans.

In the following experiments the CAMPs inhibitor was tested for its antiviral action in two cell lines. One cell line

was cultured Burkitt tumour lymphoblasts (strain Raji) infected with Epstein-Barr virus (EBV) in vitro (Kottaridis et al, J. Natl. Cancer Inst. 1977, 59(1), 89-91) and the other P3HR-1 Burkitt lymphoma, EBV producing cells (Hinuma Y. et al, 1967, J. Virol. 1, 1045-1051). IgG antibodies were demonstrated by immunofluorescence (Gull labs) on EBV-infected Raji cell smears with 10% and on P3HR-1 with 20-25% of the cells showing fluorescence. For negative controls IgG-EBV negative antibodies were used.

For chemosensitivity testing 200,000 cells/tube were used. The results for EBV-infected Raji cells showed 22% cytotoxicity at 2 U/ml CANPs inhibitor, 97% at 4 U/ml and 100% at 5 U/ml. In the case of P3HR-1, EBV producing cells, there was 95% cytotoxicity at 2 U/ml CANPs inhibitor, 100% at 4 U/ml and 5 U/ml. Both cell lines were found free of detectable immunofluorescent IgG after treatment of cells with higher than 4 U/ml CANPs inhibitor.

Although both cell lines are sensitive to the CANPs inhibitor, because they are malignant, the disappearance of immunofluorescent IgG after treatment documents the antiviral action of the CANPs inhibitor.

2) On Human Immunodeficiency Virus Type 1 (HIV-1, AIDS Virus)

The cell line MOLT-4 (ATCC CRL 1582, from acute lymphoblastic leukemia) infected with HIV-1 (Koyanagi Y.S. et al., 1987, Science 236, 819-822 and Cann A.J. et al., 1990, J. Virol. 64 (10) 4735-4 742) was used. For chemosensitivity testing 200,000 cells/tube were treated with the inhibitor of CANPs at 4 U/ml and 10 U/ml. The results showed 97% cytotoxicity at 4 U/ml and 100% at 10 U/ml inhibitor of CANPs.

Cell smears, fixed in cold acetone, were used for immunofluorescent tests, using positive human serum containing antibodiesto HIV-1 (1:100) and antihuman IgG fluorescent conjugate (1:200 Dako corp.). For negative controls human serum negative to HIV-1 antibodies was used. Immunofluorescent HIV-1-antigen was detected in 60% of untreated MOLT-4 HIV-1 infected cell cultures. The treated MOLT-4 HIV-1 infected cells were free of detectable immunofluorescent HIV-1-antigen at concentrations of 4 U and 10 U/ml inhibitor of CAMPs.

It is concluded that HIV-1 infected MOLT-4 cells are highly sensitive to the inhibitor of CAMPs for they are malignant cells but the disappearance of immunofluorescent HIV-1 -antigen documents the anti-AIDS action of the inhibitor of CAMPs.

CLAIMS

1. The use of inhibitors of calcium activated neutral proteinases (CANPs) for the manufacture of a pharmaceutical preparations for the treatment of tumours, especially cancer.

2. The use of inhibitors of calcium activated neutral proteinases (CANPs) for the manufacture of a pharmaceutical preparation for the treatment of viral diseases.

3. The use of inhibitors of calcium activated neutral proteinases (CANPs) for the manufacture of a pharmaceutical preparation for the treatment of AIDS.

4. The use of inhibitors of calcium activated neutral proteinases (CANPs) for the manufacture of anticonceptiva.

5. The use claimed in claims 1-4, wherein the optionally purified inhibitors are tetrameric proteins of MW of approximatively 240,000, based on its elution from Sephadex G-200, heatstable at neutral pH, destroyed on digestion with trypsin, and dissociated into its subunits of a MW of approximatively 60,000 by 0.1-1 mM Ca^{2+}, based on SDS-polyacrylamid gel electrophoresis, pharmaceutically acceptable addition salts, derivatives, analogues, active subunits, fragments or related compounds thereof.

6. The use as claimed in claims 1-4, wherein the inhibitors of claim 5 are endogenous native inhibitors isolated from a biological source, like erythrocytes, brain, cardia muscle, lung, spleen, liver, skeletal muscle, kidney, testis or the like, and optionally purified, but especially from rabbit skeletal muscle or liver.

7. The use as claimed in claims 1-4 of an inhibitor from rabbit skeletal muscle according to claim 5, which is manufactured and sold by Sigma Chemical Company, St. Louis, USA, under the product number P-0787.

8. The use claimed in claims 1-4, wherein the inhibitors of claims 5-7, active fragments, subunits of e.g. MW 60,000, derivatives, analogous related compounds or its pharmaceutically acceptable addition salts are produced synthetically, especially by bio- or gentechnological methods, e.g. by expression in Escherichia coli.

9. The use of a pharmaceutical preparation according to claims 1-8 in form of a solution, powder, injection, tablet, capsule, pellets, in a fast or sustained release form comprising an effective amount of an inhibitor and suitable excipients.

10. A method for the treatment of tumour, especially cancer, by administering to a human or warmblooded animal an effective amount of a CAMP inhibitor as claimed in claims 1-9.

11. A method for the treatment of viral infections by administering to a human or warmblooded animal an effective amount of a CAMP inhibitor as claimed in claims 1-9.

12. A method for the treatment of AIDS by administering to a human an effective amount of a CAMP inhibitor as claimed in claims 1-9.

13. A method for the treatment against fertilization by administering to a human or warmblooded animal an effective amount of a CAMP inhibitor as claimed in claims 1-9.

14. A method according to claims 10-13, characterized by administering the effective amount of a CAMP inhibitor

intramuscularly, subcutaneously, intraperitoneally or intravenously.

15. A method according to claims 10-14, wherein the effective amount of an inhibitor is of 1 mg/kg body weight per day to 25 mg/kg body weight per day.

16. A pharmaceutical preparation manufactured according to any of the preceding claims.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 92/01223

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K37/64

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOLOGICAL ABSTRACTS vol. 68 , 1979, Philadelphia, PA, US; abstract no. 16423, NISHIURA, IWAO ET AL. 'THE ROLE OF INTRACELLULAR PROTEASES IN BRAIN TUMOR.' see abstract & NEUROL. MED-CHIR vol. 19, no. 1, 1979, pages 1 - 8	1
A	WO,A,9 000 401 (UNIVERSITY COLLEGE LONDON) 25 January 1990	

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 OCTOBER 1992	02.11.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	REMPP G.L.E.

Form PCT/ISA/210 (second sheet) (January 1985)